

Peptide Handling by HLA-B27 Subtypes Influences Their Biological Behavior, Association with Ankylosing Spondylitis and Susceptibility to Endoplasmic Reticulum Aminopeptidase 1 (ERAP1)*[§]

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HLA-B27 is strongly associated with ankylosing spondylitis (AS). We analyzed the relationship between structure, peptide specificity, folding, and stability of the seven major HLA-B27 subtypes to determine the role of their constitutive peptidomes in the pathogenicity of this molecule. Identification of large numbers of ligands allowed us to define the differences among subtype-bound peptidomes and to elucidate the peptide features associated with AS and molecular stability. The peptides identified only in AS-associated or high thermostability subtypes with identical A and B pockets were longer and had bulkier and more diverse C-terminal residues than those found only among non-AS-associated/lower-thermostability subtypes. Peptides sequenced from all AS-associated subtypes and not from non-AS-associated ones, thus strictly correlating with disease, were very rare. Residue 116 was critical in determining peptide binding, thermodynamic properties, and folding, thus emerging as a key feature that unified HLA-B27 biology. HLA-B27 ligands were better suited to TAP transport than their N-terminal precursors, and AS-associated subtype ligands were better than those from non-AS-associated subtypes, suggesting a particular capacity of AS-associated subtypes to bind epitopes directly produced in the cytosol. Peptides identified only from AS-

associated/high-thermostability subtypes showed a higher frequency of ERAP1-resistant N-terminal residues than ligands found only in non-AS-associated/low-thermostability subtypes, reflecting a more pronounced effect of ERAP1 on the former group. Our results reveal the basis for the relationship between peptide specificity and other features of HLA-B27, provide a unified view of HLA-B27 biology and pathogenicity, and suggest a larger influence of ERAP1 polymorphism on AS-associated than non-AS-associated subtypes. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M114.039214, 3367–3380, 2014.

The current ideas concerning the pathogenetic role of HLA-B27 in ankylosing spondylitis (AS) emphasize specific antigen presentation (1), misfolding (2), or immunomodulation mediated by heavy chain homodimers (3) expressed at the cell surface upon endosomal recycling (4). Recent research provided evidence that both misfolded HLA-B27 heavy chains and surface expressed B27 homodimers may activate the IL-23/IL-17 axis, a key inflammatory pathway in spondyloarthropathies, through distinct mechanisms, namely the unfolded protein response (5) and the stimulation of IL-17-producing T cells (6). In contrast, the fact that CD8⁺ T cells are not required for the HLA-B27-associated disease in transgenic rats (7, 8), and the failure to identify specific *arthritogenic* peptides, point out to a pathogenetic role of HLA-B27 based on its folding and/or non-canonical forms, rather than to an autoimmune mechanism based on molecular mimicry between foreign and self-derived peptides. Yet, on the basis of genetic and immunological studies (9, 10), an involvement of CD8⁺T cells in the human disease cannot be ruled out.

Beyond the pathogenetic relevance of specific peptides, the constitutive HLA-B27-bound peptidome is related to the folding and stability of HLA-B27, because both features are peptide-dependent (11). This is strongly supported by the association of ERAP1, an aminopeptidase that trims peptides to their optimal size for MHC-I binding (12, 13), with ankylos-

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ing spondylitis (AS)¹ among HLA-B27-positive individuals (14), and by the demonstration that AS-associated ERAP1 polymorphism has a substantial effect on the HLA-B27 peptidome in live cells (15).

Any pathogenetic mechanism must account for the differential association of HLA-B27 subtypes with AS. Whereas B*27:02, B*27:04 and B*27:05 are clearly associated with this disease, B*27:06 and B*27:09 are not (16, 17). B*27:07, a subtype present in multiple populations, is generally associated with AS, with one reported exception (18, 19). All these subtypes have the same structure in the A and B pockets of their peptide binding site, which accommodate the two N-terminal residues of their peptide ligands, but they differ in one or more positions in the F pocket, which binds the C-terminal peptide residue, as well as in other positions of the peptide binding site. In contrast, B*27:03, a subtype prevalent only in populations of Sub-Saharan African ancestry, differs from the B*27:05 prototype by a single Y59H change in the A pocket (20, 21), a difference that also sets it apart from all other subtypes (supplemental Table S1) and affects the binding preferences for N-terminal peptide residues (22–24). The nature of B*27:03 as a putative susceptibility factor for AS is unclear (19). In African populations in which this subtype is prevalent, neither this subtype nor B*27:05 are associated with this disease (25), presumably because of concurrent protective factor(s).

In this study we carried out an extensive sequence analysis of HLA-B27 subtype-bound peptidomes to define their differential features as well as the extent and nature of peptide sharing among subtypes. The results revealed the basis for the intimate relationship between peptide specificity, folding, and stability of HLA-B27, provided a unified explanation on how subtype polymorphism alters the molecular biology of HLA-B27 and its association with AS, and demonstrated a differential influence of TAP and ERAP1 on AS-associated and non-AS-associated subtypes.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—HMy2.C1R (C1R) is a human lymphoid cell line expressing low levels of its endogenous HLA-I antigens (26). C1R transfectants expressing HLA-B*27:02, 03, 04, 05, 06, 07, or 09 (24, 27–30) were used. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Paisley, UK). The W6/32 monoclonal antibody (IgG2a; specific for a monomorphic HLA class I determinant) (31) was used for immunopurification of HLA-B27.

Isolation of HLA-B27-bound Peptidomes—This was carried out as previously described (32) with some modifications. About 5×10^9 cells were lysed in 1% Igepal CA-630 (Sigma-Aldrich, St Louis, MO) in the presence of a mixture of protease inhibitors (Roche, Mannheim, Germany). HLA-B27/peptide complexes were purified from the soluble fraction by affinity chromatography using the W6/32 antibody and

eluted with 0.1% trifluoroacetic acid (TFA). HLA-B27 ligands were isolated by filtering with Centricon 3 (Amicon, Beverly, MA), concentrated in SpeedVac and subjected to reverse phase HPLC chromatography in a Waters Alliance 2690 instrument (Waters, Milford, MA) using a Vydac 218TP52-C18 column (Vydac, Hesperia, CA) as previously described (33). The eluted peptides were collected and stored at -20°C .

Electrospray-Orbitrap Mass Spectrometry Analysis—Peptide sequencing by Electrospray-Orbitrap was performed exactly as described elsewhere (34) by $\mu\text{LC-MS/MS}$ using an Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA) fitted with a capillary HPLC (Eksigent, Dublin, CA).

Electrospray-Q-TOF Mass Spectrometry Analysis—The peptide pools eluted from B*27:03, B*27:06, or B*27:09 were additionally analyzed in a nano-LC Ultra HPLC (Eksigent, AB Sciex, Redwood City, CA) coupled online with a 5600 triple TOF mass spectrometer (AB Sciex) through a nanospray III source (AB Sciex) exactly as described elsewhere (35).

Database Searches—Peptide sequences obtained by Electrospray-Orbitrap were identified as follows. Pep-Miner (36) was used for generation of the peak-lists based on the $\mu\text{LC-MS/MS}$ data. The peptides were identified using multiple search engines: Pep-Miner, Sequest (Thermo-Fisher) (37) and Mascot (server 2.2, Matrix Science Inc. Boston, USA) (38), and searched against the human part of the Uniprot database (<http://www.uniprot.org>, May 2011) including 20,381 proteins. The Sequest and Mascot search results were combined into one report by Proteome Discoverer 1.3 (Thermo-Fisher). The search was not limited by enzymatic specificity, the peptide tolerance was set to 0.01 Da and the fragment ion tolerance was set to 0.5 Da. Oxidized M was searched as a variable modification. Peptide identifications were accepted if their masses were below 1500 Da and mass accuracy was better than 0.005 Da. Other required criteria were: Pep-Miner Score ≥ 85 , Sequest Xcore 2.0, Sequest Probability ≤ 0.05 . Some peptides were also observed with different charge-states and as overlapping, longer or shorter peptides by one amino acid, listed in the table as variants. The false discovery rate (FDR) of the peptides adhering to four or more of these criteria was $<5\%$. Similar data analysis was also carried out with the MaxQuant software (39). MaxQuant (version 1.4.1.2) was used with the Andromeda search engine and the human section of the UniProt-proteome/Swiss-Prot database (release 2013_12) including 69,001 proteins. Peptide precursor and fragment mass tolerance were set at 6 ppm and 0.5 Da, respectively. The FDR was set to $<5\%$.

In the experiments carried out using the 5600 triple TOF mass spectrometer, raw data were processed with the Protein Pilot software (version 4.5) to generate a recalibrated mgf file that was used in a MS/MS search with MASCOT (version 2.4) as search engine. A concatenated target-decoy protein database was generated by combining the Uniprot complete human proteome set (downloaded on May 23, 2011) with its corresponding reversed database generated with the DBToolKit software (version 4.1.4). Search parameters were set as follows: No enzyme, peptide tolerance: 10 ppm, MS/MS tolerance: 25 mDa, instrument: ESI-QUAD-TOF, and variable modifications: dioxidation of C, oxidation of M and pyroglutamic acid formation from N-terminal Q. Only the peptide sequences that fitted the canonical B27 binding motif (R or Q at P2) with a FDR $< 5\%$ at the peptide level were considered. FDR was estimated by decoy hit counting as previously described (40, 41).

Statistical Analyses—Except when otherwise stated the χ^2 test with Bonferroni's correction was used. Statistical significance was assessed at $p < 0.05$.

Analysis of Protein Features—Molecular weights and isoelectric point (pI) of the precursor proteins and the human proteome (UniProtKB database release 2011.1: 2012/11/28) were obtained with the

¹ The abbreviations used are: AS, ankylosing spondylitis; C1R, HMy2.C1R; FDR, false discovery rate; RF, residue frequency; TFA, trifluoroacetic acid.

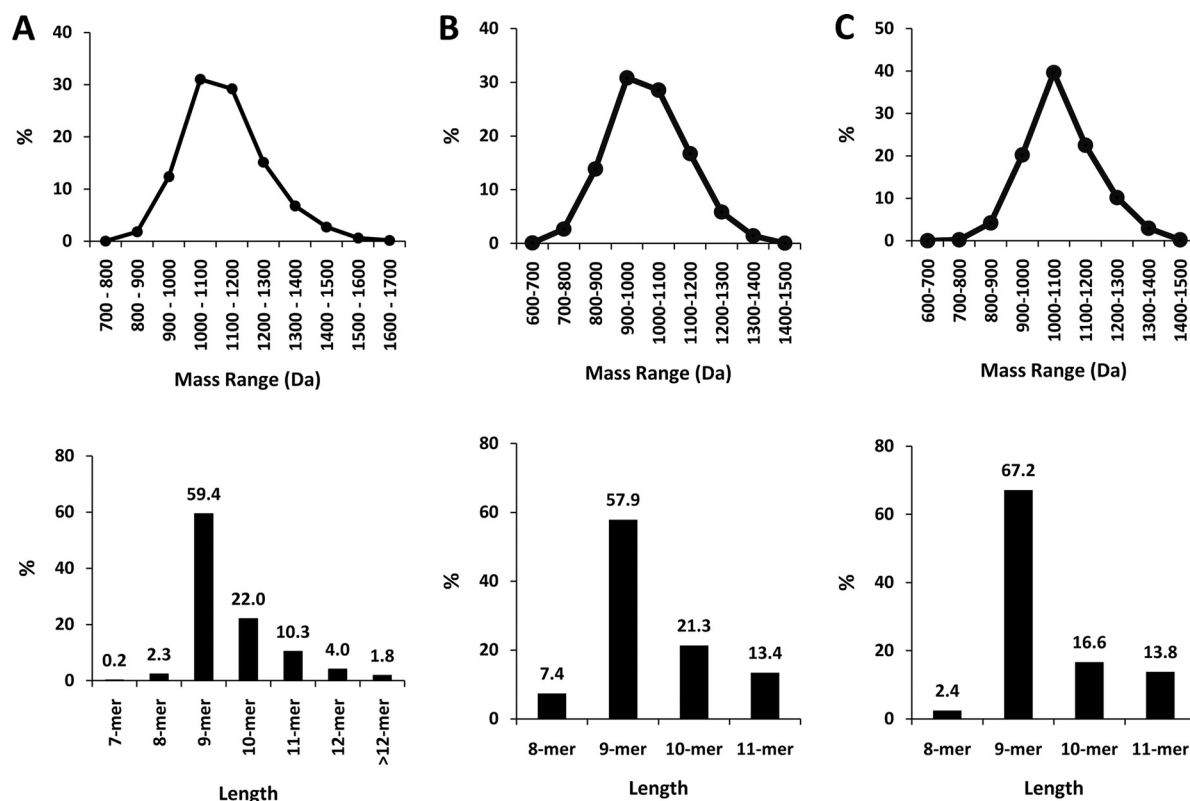


FIG. 1. Mass range (upper panel) and length distribution (lower panel) of 2229 HLA-B27-bound and other peptides. A, The HLA-B27 ligands are listed in [supplemental Table S2](#). Five peptides with MW>1700 Da are not included in the upper panel. B, Mass range (upper panel) and length distribution (lower panel) of HLA-B7 (B*07:02) ligands. C, Mass range (upper panel) and length distribution (lower panel) of HLA-A2 (A*02:01) ligands. Data from panes B and C are based on previously published peptide databases (45) and are included here only for comparison. These series included only peptides up to 1500 Da and 8–11 residues.

ExPASy compute pI/Mw tool (http://web.expasy.org/compute_pi/). Subcellular locations were obtained by using the Protein Information and Knowledge Extractor tool (42).

TAP-binding Affinity—This was estimated for HLA-B27 ligands and their N-terminally extended precursors using the TAPREG tool (43), which makes use of support vector machines trained with large peptide sets to predict the TAP binding affinity of peptides of eight to 16 residues. Statistical significance between peptide sets was assessed with the Mann-Whitney test.

Estimation of ERAP1 Sensitivity—ERAP1 susceptibility scores for N-terminal flanking and P1 residues were calculated as previously described (15). The method assigned a score, ranging from 0 to 100, to each amino acid, based on its susceptibility to ERAP1, as established in experimental trimming assays (44).

RESULTS

HLA-B27-bound Peptidomes and their Parental Proteins—

The constitutive peptidomes of B*27:02, B*27:03, B*27:04, B*27:05, B*27:06, B*27:07, and B*27:09 were subjected to MS/MS sequencing using Electrospray-Orbitrap MS and both Proteome Discoverer and MaxQuant-based peptide identifications. The peptidomes of B*27:03, B*27:06, and B*27:09 were additionally subjected to Triple TOF MS analyses. Only peptides up to 18 residues long and showing either R or Q at position 2 were included in this study. With these data 2229 peptide sequences ([supplemental Table S2](#)) were compiled

from the following four sources: 1) electrospray-orbitrap MS analyzed using Pep-Miner, Sequest, Mascot, and the Protein Discoverer software ([supplemental Table S3](#)), 2) electrospray-orbitrap MS data re-analyzed using MaxQuant, which significantly increased the number of assignments both of new ligands and of known ligands to additional subtypes ([supplemental Table S4](#)), 3) B*27:03, B*27:06, and B*27:09 ligands identified by electrospray-Q-TOF MS ([supplemental Table S5](#)), and 4) previously published ligands from multiple subtypes. This approach precludes quantitative comparisons and leaves open the possibility that peptides not found in a given subtype may still bind to it in some amount. Yet, we were able for the first time to identify sufficiently high numbers of peptides from multiple HLA-B27 subtypes to perform a reliable comparison of their peptidomes and of their main distinctive features.

This total set, which was used for all subsequent analyses, encompassed 1523 novel HLA-B27 ligands and many others that were previously reported from some of the subtypes and were found now in additional ones. These ligands arose from at least 2092 proteins, whose MW, pI, and subcellular distribution were similar to the human proteome, except for a sub-representation of ligands arising from membrane proteins ([supplemental Fig. S1](#)). The MW of this whole peptide set

TABLE I
HLA-B27 subtype-bound ligands

Subtypes	^a N	^b Specific	^b Shared	^c Mean MW	^d Mean length	<9-mers ^e	9-mers ^e	10-mers ^e	>10-mers ^e
Total	2229	–	–	1130.9 ± 133.2	9.6 ± 1.1	2.5	59.4	22.0	16.1
B*27:02	1120	20.2	79.8	1149.7 ± 133.8	9.8 ± 1.1	0.4	53.6	27.0	19.0
B*27:03	510	18.4	81.6	1146.6 ± 139.7	9.7 ± 1.2	0.8	60.6	21.6	17.1
B*27:04	1046	24.9	75.1	1115.7 ± 123.5	9.4 ± 1.0	2.3	68.7	18.6	10.3
B*27:05	1120	16.1	83.9	1145.1 ± 133.6	9.7 ± 1.1	1.3	54.6	25.0	19.2
B*27:06	555	18.4	81.6	1071.3 ± 118.3	9.3 ± 0.9	2.0	79.8	11.5	6.7
B*27:07	768	9.1	90.9	1116.1 ± 128.8	9.6 ± 1.0	1.3	61.6	22.3	14.8
B*27:09	743	12.8	87.2	1110.6 ± 132.6	9.6 ± 1.0	0.5	65.5	19.9	14.0

^aTotal number of sequenced peptides.
^bPercentage of peptides found exclusively in the corresponding subtype (specific) or in at least another subtype (shared), respectively.
^cMean mass value (Da) in single-charge state (M+H⁺).
^dMean number of residues.
^ePercent values relative to the total amount of sequences in the corresponding subtype.

showed a Gaussian distribution centered on 1000–1200 Da and somewhat deformed at the high MW end, reflecting a higher tolerance for peptides of high MW, relative to small ones. A majority of the peptides were nonamers, followed by decamers and longer peptides (Fig. 1A). This pattern was similar to that found among HLA-B*07:02 ligands and only slightly different from HLA-A*02:01 (Fig. 1B–1C) in a previous study (45).

Subtype-bound Peptidomes: General Features—The total number of peptide sequences from each subtype is shown in Table I. Differences were found in the mean MW (ranging from 1071.3 to 1149.7 Da) and length (9.3 to 9.8 residues) of subtype-bound peptidomes. The length of peptides from individual subtypes showed some significant differences. For instance, 9-mers ranged from 53.6/54.6% to 79.8% ($p: 1.2 \times 10^{-24}/4.8 \times 10^{-23}$), 10-mers from 27.0/25.0 to 11.5% ($p: 4.0 \times 10^{-12}/8.2 \times 10^{-10}$), and peptides >10-mers from 19.0/19.2% to 6.7% ($p: 1.6 \times 10^{-10}/9.6 \times 10^{-11}$) for B*27:02/B*27:05 and B*27:06, respectively. Other subtypes showed intermediate values. The percent of peptides found only in one subtype ranged from 9.1 to 24.9% (B*27:07/B*27:04), indicating a large overlap among subtype-bound peptidomes.

Subtype-bound Peptidomes: Residue Usage—The comparative analysis of residue frequencies (RF) among subtype-bound peptide repertoires was carried out separately for main anchor (P2 and PC), secondary anchor (P1, P3, and PC-2) and “nonanchor” positions (P4–P6, and P8). *N*-fold and statistical significance of relevant differences are shown in supplemental Table S6.

Main Anchor Positions (Fig. 2)—

P2—As expected from the identity of the B pocket, the overwhelming majority of peptides from any given subtype had R2. In addition, Q2, previously reported only in B*27:05 (46–48), was found with low frequency (1.8 to 3.3%) in all subtypes.

PC—F and L were among the most frequent C-terminal residues in all subtypes (RF: 15.4–29.8% and 23.3–57.1%, respectively). Other aliphatic residues were less frequent. Y

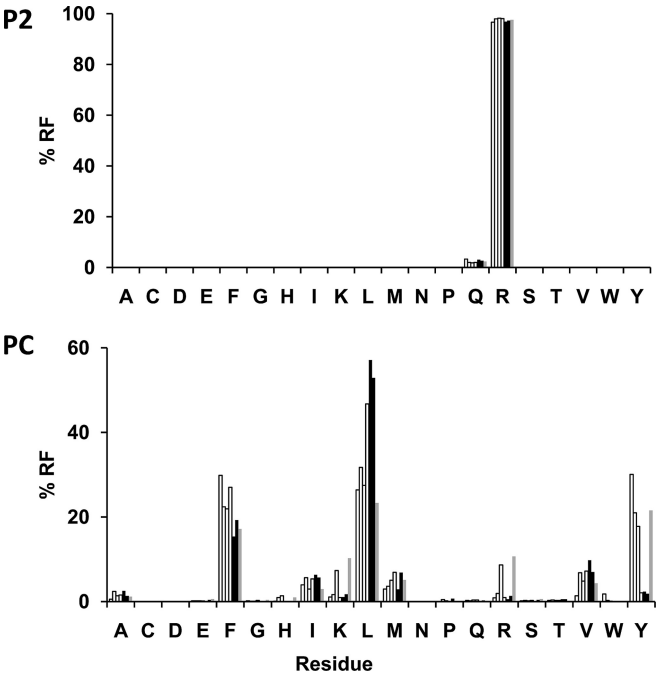


FIG. 2. Residue frequencies (% RF) among subtype-bound HLA-B27 ligands at their main anchor positions P2 and PC. AS-associated (B*27:02, 04, 05, and 07) and non-AS-associated (B*27:06 and 09) subtypes are shown, and in that order, with white and black bars, respectively. B*27:03 is shown with a gray bar. For *n*-fold and statistical significance of relevant differences see supplemental Table S6.

was frequent in most AS-associated subtypes (B*27:02, 04, 05) and B*27:03 (17.8–30.1%), but rare in B*27:06, 07, and 09 (1.9–2.4%). Basic residues (K, R) were frequent only in B*27:03 and 05 (7.3–10.7%). Other residues were rare or absent altogether. Significant differences in both the chemical nature and size of the C-terminal peptide residues were observed among subtypes, as a function of residue 116 (Table II and supplemental Table S6). Subtypes lacking D116 (B*27:06, 07, and 09) showed an increased frequency of aliphatic residues relative to subtypes with D116 (67.9–78.7% versus 35.2–50.2%), an almost absolute restriction (>90%) to non-

TABLE II

Polarity and size of C-terminal residues among HLA-B27 subtype-bound peptides. The nature of residue 116 in each subtype is indicated. Figures are residue frequencies (%) among the peptides sequenced from the corresponding subtype. Values selectively increased in a subtype group are highlighted in boldface. For the statistical significance of the differences in residue usage among subtypes in the C-terminal position see [supplemental Table S6](#)

	B*27:02 (D116)	B*27:03 (D116)	B*27:04 (D116)	B*27:05 (D116)	B*27:06 (Y116)	B*27:07 (Y116)	B*27:09 (H116)
POLARITY							
Basic	2.1	21.9	4.5	17.4	1.6	1.8	3.1
Non-polar Aliphatic	35.2	37.1	50.2	41.6	78.7	67.9	74.0
Non-polar Aromatic (F)	29.8	17.2	22.4	21.9	15.4	27.0	19.3
Polar Aromatic (Y)	30.1	21.5	21.0	17.7	2.4	2.1	1.9
SIZE (Da)							
57–103	2.5	7.0	10.4	6.9	13.9	9.6	9.6
104–115	30.3	26.3	37.4	30.4	63.7	52.1	58.6
116–137	4.6	17.2	6.6	14.2	4.2	8.4	9.3
138–186	62.6	49.6	45.6	48.4	18.3	30.0	22.5

TABLE III

Summary of HLA-B27 subtype features

Subtype	AS association	Residue 116	Folding ^a	Thermostability ^a	PC residues
B*27:02	Yes	D	Slow	High	Y, Nonpolar
B*27:03 ^b	Not known	D	Fast ^b	Low ^b	Basic, Y, Nonpolar
B*27:04	Yes	D	Slow	High	Y, Nonpolar
B*27:05	Yes	D	Slow	High	Basic, Y, Nonpolar
B*27:06	No	Y	Fast	Low	Nonpolar
B*27:07	Yes	Y	Fast	Low	Nonpolar
B*27:09	No	H	Fast	Low	Nonpolar

^a Data from (58).

^b B*27:03 differs from all other subtypes by the Y59H change in the A pocket. The influence of this change on folding and thermostability sets B*27:03 apart from other subtypes in the correlation of these features with residue 116.

polar aliphatic residues and F, and a preference for small residues (104–115 Da: 52.1–63.7%). Subtypes with D116 were permissive to C-terminal Y (17.7–30.1%) and, some of them (B*27:03, 05), to basic residues (21.9 and 17.4%, respectively), and showed an increased preference (45.6–62.6%) for bulky (>138 Da) C-terminal residues.

There was a close correlation between the structure, folding, and thermostability of HLA-B27 subtypes with identical A and B pockets, therefore excluding B*27:03, and their C-terminal motifs (Table III). These subtypes could be classified in two groups: (1) D116/slow folding/high thermostability/allowance for Y/preference for bulky C-terminal residues (B*27:02, 04, and 05), and (2) non-D116/fast folding/low thermostability/high restriction to nonpolar C-terminal residues, almost excluding Y/preference for C-terminal residues of moderate size (B*27:06, 07, and 09).

Secondary Anchor Positions (Fig. 3)—

P1—The most frequent residues at this position were A, G, H, K, and S. They showed comparable frequencies in all subtypes except B*27:03, which had statistically increased H and K, and decreased G and A frequencies, relative to other subtypes. R was also frequent and most abundant in B*27:03 and 04 ([supplemental Table S6](#)). Other residues were used with smaller and similar frequencies in all subtypes.

P3—The most frequent residues at this position were L and F, although many other residues were allowed. B*27:04 and B*27:06 showed slightly increased frequencies of basic P3 residues (R, K), relative to other subtypes. In contrast, B*27:03 showed a particularly low allowance for K and H ([supplemental Table S6](#)).

PC-2—Restrictions at this position were low, allowing for both polar and nonpolar side chains. Residue usage was generally similar among subtypes, with some exceptions. For instance, L and K were statistically increased in B*27:04 and B*27:06, respectively, R was increased and E was decreased in both of these subtypes, V was decreased in B*27:06, relative to most other subtypes ([supplemental Table S6](#)).

“Nonanchor” Positions ([supplemental Fig. S2](#))—Because a comparison of the central peptide positions requires a precise alignment of peptide sequences, this analysis can only be properly done with peptides of the same length and was carried out only for positions P4–P6 and P8 of nonamers. Restrictions were low at all four positions, but each one showed some statistically significant bias toward particular residues as well as individual subtype preferences ([supplemental Table S6](#)). The most frequent residues at P4 were P (15.9–20.8%) and G (12.4–14.0%) for all subtypes. At P5 the most frequent residues were G (7.2–11.3%), L (8.2–11.4%),

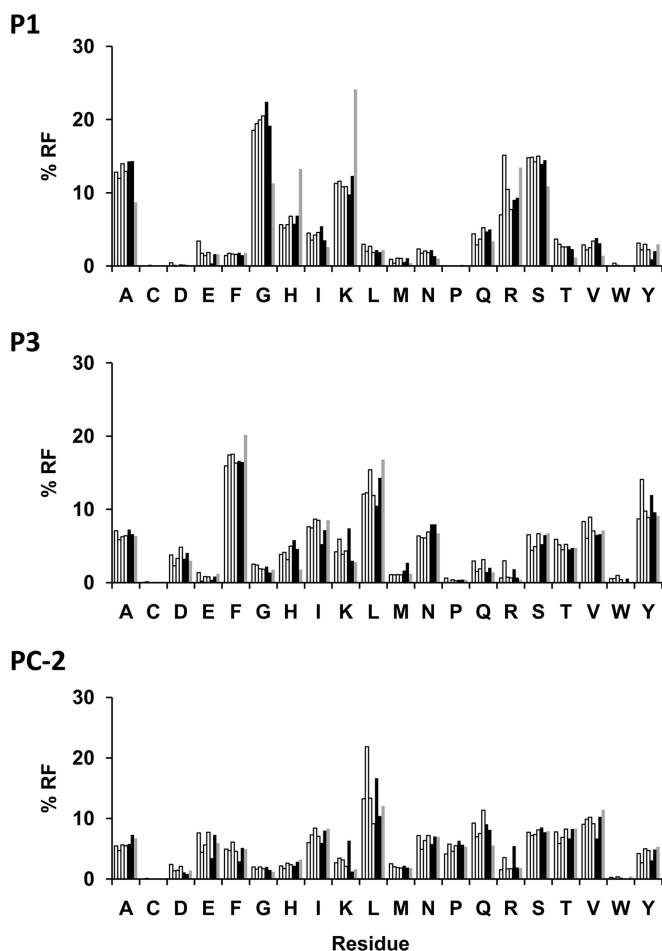


FIG. 3. Residue frequencies (% RF) among subtype-bound HLA-B27 ligands at their secondary anchor positions P1, P3, and PC-2. B*27:02, 04, 05 and 07 are shown, in that order, with white bars, B*27:06 and 09 are shown with black bars and B*27:03 is shown with a gray bar. For *n*-fold and statistical significance of relevant differences see supplemental Table S6.

and V (5.8–12.6%); the two latter residues were less frequent in B*27:06 and B*27:04, respectively. In contrast, R5 was more frequent in these (2.7–4.6%) than in other subtypes (0.3–1.8%). At P6 the most frequent residues were I, L, V, and polar residues (*i.e.* Q, S), with RF ≈ 10% each in all subtypes at P8 the most frequent residues were A, E, G, K, L, N, Q, S, and T. Some differences among subtypes were observed, most notably concerning the lower frequency of N (0.5–0.6-fold) and higher frequency of V (about twofold) in B*27:04 and B*27:06. These results indicate that subtype polymorphism has an influence on residue usage also at nonanchor positions.

Peptide Sharing Among HLA-B27 Subtypes—In previous studies we carried out a series of pairwise comparisons between subtype-bound peptide repertoires (29, 30, 49, 50). These studies were based on identity of MW, chromatographic retention time, and limited sequencing. Here, we reassessed the overlap among subtype-bound peptidomes

TABLE IV
Length and MW of peptide ligands from subtypes with differential AS association or thermostability

AS association	N	Mean length	Mean MW
^a AS-associated	1167	9.7 ± 1.1	1159.5 ± 130.1
^b Non-AS associated	227	9.2 ± 0.8	1041.6 ± 110.5
Thermostability			
^c High	988	9.7 ± 1.1	1163.7 ± 130.5
^d Low	313	9.3 ± 0.9	1058.5 ± 116.1

^a Peptides found in one or more of the AS-associated subtypes (B*27:02, 04, 05, 07) but not in the non-AS-associated ones (B*27:06, 09).

^b Peptides found in one or more of the non-AS-associated subtypes but not in the AS-associated ones.

^c Peptides found in one or more of the high thermostability subtypes (B*27:02, 04, 05) but not in those with lower thermostability (B*27:06, 07, 09).

^d Peptides found in one or more of the low thermostability subtypes but not in those with high thermostability.

based on the many novel ligands sequenced from distinct subtypes (supplemental Table S7). All subtype pairs showed similar levels of peptide sharing (about 60–74%). Of 2229 HLA-B27 ligands, 1008 (45.2%) were found only in one subtype and the remaining peptides showed highly diverse patterns of subtype distribution (supplemental Table S2).

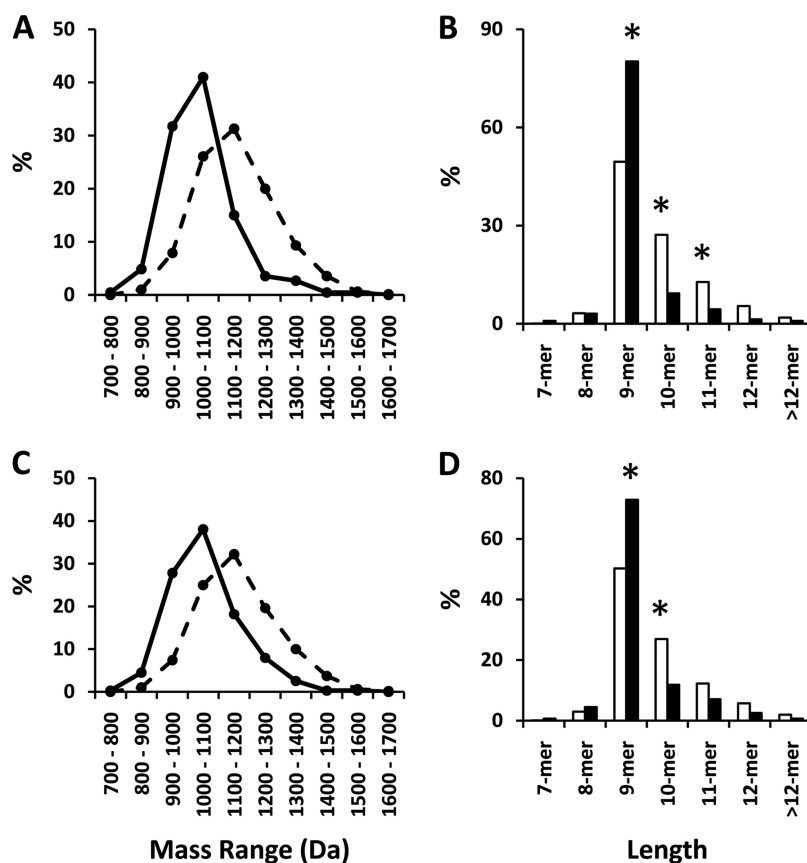
Peptides Selectively Presented by AS-associated or non-AS-associated Subtypes—A total of 1167 ligands were identified from one or more AS-associated subtypes, but were not found in the non-AS-associated ones. Likewise, 227 peptides were found only in the non-AS-associated B*27:06 and/or B*27:09 subtypes (Table IV). The mean length and MW of the former peptide set were bigger by 0.5 residues and more than 100 Da, respectively, relative to the latter one. A clear shift was observed in the modal distribution of MW and length between both peptide sets: Heavier and longer ligands were more abundant in the AS-associated subtypes (Fig. 4A–B). Of note, 9-mers accounted for 80.2% of the peptides selectively found among non-AS-associated subtypes compared with only 49.5% in AS-associated ones ($p = 1.6 \times 10^{-16}$).

Significant differences in residue usage at the C-terminal position, concerning both polarity and size, were also found between both subsets (Fig. 5A–5C). Bulky aromatic and basic residues were much more frequent in the AS-associated set. In contrast, small and aliphatic residues, especially L, prevailed among the peptides found only in non-AS-associated subtypes.

These results indicate that AS-associated and non-AS associated subtypes selectively bind peptides of distinct length and MW, which are distinguished by differential use of their prevalent C-terminal residues.

Peptides Selectively Presented by HLA-B27 Subtypes with High or Low Thermostability—A total of 988 and 313 ligands were found only among high (B*27:02, 04, and 05) or low (B*27:06, 07, and 09) thermostability subtypes, respectively (Table IV). B*27:03 was not included in this analysis because

FIG. 4. MW and length of peptide subsets correlating with AS or molecular stability. A, Mass range distribution of peptides found exclusively in AS-associated (dashed line) or non-AS-associated (solid line) HLA-B27 subtypes. B, Length distribution of peptides found exclusively in AS-associated (white bars) or non-AS-associated (black bars) subtypes. Statistically significant differences in the frequency of 9-mers, 10-mers and 11-mers in both peptide sets (marked with *) were observed ($p = 1.6 \times 10^{-16}$, 5.4×10^{-8} , and 1.7×10^{-3} , respectively). C, Mass range distribution of peptides found exclusively in high (dashed line) or low (solid line) HLA-B27 thermostability subtypes. D, Length distribution of peptides found exclusively in high (white bars) or low (black bars) thermostability subtypes. Statistically significant differences in the frequency of 9-mers and 10-mers in both peptide sets (marked with *) were observed ($p = 1.4 \times 10^{-11}$ and 2.2×10^{-7} respectively).



its low thermostability is due to a change in the A pocket and, therefore, has a different basis relative to the other subtypes. The peptides found only in the high thermostability subtypes had higher mean length (by 0.4 residues) and MW (by 105 Da) than those found only in the low thermostability subtypes (Table IV) and the MW and length distribution of both subsets (Fig. 4C–4D) indicated that peptides heavier and longer were more abundant in the former one. Bulky aromatic and basic C-terminal residues were preferentially found in high thermostability subtypes, whereas L and other small aliphatic residues were predominant in the low thermostability subset (Fig. 5D–5F).

These results indicate that the differential features between peptides associated with high or low thermostability are similar to those distinguishing the peptides found only among AS-associated or non-AS-associated subtypes. High/low thermostability and AS-/non-AS-associated subtype groups differ only by B*27:07, which is AS-associated but shows low thermostability. Because of this, the peptides found only among high thermostability subtypes were less than those found only among AS-associated subtypes (988 compared with 1167), and the peptides found only among the low thermostability subtypes were increased relative to non-AS-associated subtypes (313 compared with 227). Yet, this was not sufficient to alter the similarity of peptide features between

AS-associated/high-thermostability or non-AS-associated/low-thermostability subtypes.

Peptides Strictly Correlating with AS Association or Thermostability are Rare—A total of 26 peptides, mostly with C-terminal F, were found in all four AS-associated subtypes and not in non-AS-associated ones. In addition, 22 peptides, all with C-terminal aliphatic residues, were found in both B*27:06 and 09 and not among AS-associated subtypes. As many as 125 ligands were exclusively found in all three high thermostability subtypes. Their predominant C-terminal motif (88%) was Y. Only two ligands, both with C-terminal L, were exclusively shared by the three subtypes with low thermostability (supplemental Table S8).

HLA-B27 Ligands are Better Suited to TAP than their Precursors—To determine how the last stages of antigen processing contribute to shape HLA-B27-bound peptidomes, the N-terminal flanking residues of these ligands in their parental proteins were analyzed for their contribution to TAP-mediated transport and to ERAP1 trimming.

The affinity of peptides for TAP depends on their whole sequence (43), but mainly on the three N-terminal and the C-terminal residues (51). The TAP-binding affinity of HLA-B27 ligands and their N-terminally extended precursors, by 1 to 4 residues, was compared using a predictive algorithm that takes into account the contribution of all peptide resi-

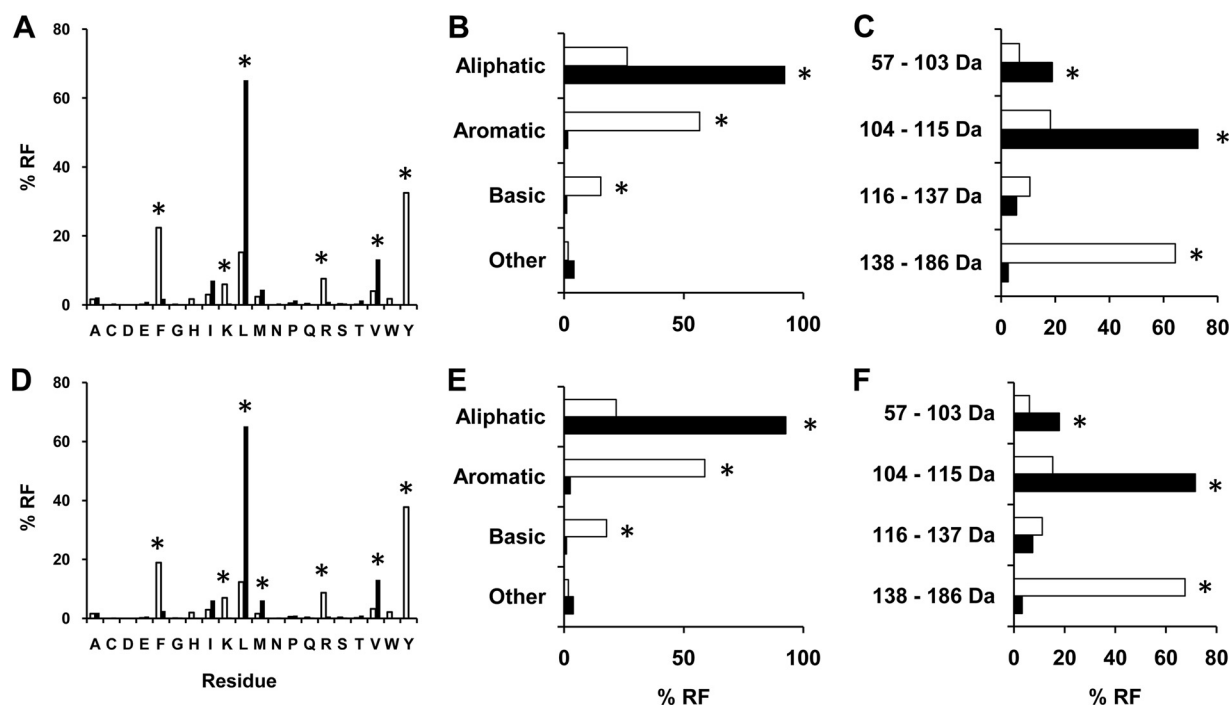


FIG. 5. C-terminal residue usage among peptide subsets correlating with AS or molecular stability. A, Residue frequencies (% RF) among peptides found exclusively in the AS-associated subtypes B*27:02, 04, 05, and 07 (white bars) or the non-AS-associated ones, B*27:06 and 09 (black bars) at the C-terminal position. Statistically significant differences ($p < 0.05$) are marked with (*). B, Chemical classification of the C-terminal residues from peptides found exclusively in the AS-associated (white bars) or non-AS-associated subtypes (black bars). Statistically significant differences (marked with *) were observed among aliphatic, aromatic and basic residues in both subsets ($p = 2.1 \times 10^{-78}$, 6.8×10^{-51} , and 7.3×10^{-8} , respectively). C, Size-based classification of C-terminal residues from peptides found exclusively in the AS-associated (white bars) or non-AS-associated subtypes (black bars). Statistically significant differences were observed among peptides in the ranges 57–103, 104–115, and 138–186 Da in both subsets ($p: 2.5 \times 10^{-8}$, 1.0×10^{-62} , and 3.0×10^{-64} , respectively). D, C-terminal residue frequencies among peptides found exclusively in the high (B*27:02, 04, and 05: white bars) or low (B*27:06, 07, and 09: black bars) thermostability subtypes. Statistically significant differences ($p < 0.05$) are marked with (*). E, Chemical classification of C-terminal residues from peptides found exclusively in the high (white bars) or low thermostability subtypes (black bars). Statistically significant differences (marked with *) were observed among aliphatic, aromatic, and basic residues in both subsets ($p = 3.8 \times 10^{-110}$, 6.9×10^{-67} , and 4.6×10^{-13} , respectively). F, Size-based classification of C-terminal residues in peptides found exclusively in the high (white bars) or low thermostability subtypes (black bars). Statistically significant differences were observed among peptides in the ranges 57–103, 104–115, and 138–186 Da in both subsets ($p = 1.4 \times 10^{-9}$, 1.3×10^{-80} , and 1.7×10^{-86} , respectively).

dues (43). The final HLA-B27 ligands were significantly more suited to TAP than any of their N-terminally extended precursors. Among these, the best ones were those extended by only one residue (Fig. 6A). This pattern is different to that found among HLA-B*07:02 or HLA-A*02:01 ligands (Fig. 6C–6D) reported in a previous study (45). Our results strongly suggest that natural HLA-B27 ligands that are generated in their final form in the cytosol will reach the ER more efficiently than their precursors.

Peptides Found Only Among AS-associated Subtypes are the Best TAP Ligands—The natural ligands selectively found among AS-associated subtypes were significantly better suited to TAP than those found only in non-AS-associated ones (Fig. 6B). Because the former set has more diverse C-terminal motifs (Fig. 5A), and these are major contributors to TAP affinity, we considered the possibility that this diversity could account for the better suitability to TAP. Thus, the peptides found only among AS-associated subtypes were

classified on the basis of their C-terminal residues and their TAP affinity was estimated separately for each subset (Fig. 6B). The results showed that the higher affinity of the AS-associated peptide set to TAP is mainly caused by the peptides with aromatic C-terminal residues, although peptides with aliphatic or basic residues were also better suited to TAP than peptides found only among non-AS-associated subtypes. A large majority of these peptides had aliphatic C-terminal residues (Fig. 5A). Thus, the fact that AS-associated peptides with aliphatic C-terminal residues were still better TAP binders indicates that residues at other peptide positions contribute to the higher affinity of this peptide subset.

Distinct Influence of ERAP1 on the Peptidomes of Subtypes with Differential AS Association and Thermostability—ERAP1 polymorphism is associated with AS (14, 52) and lack of this enzyme affects the surface expression of AS-associated, but not of non-AS-associated HLA-B27 subtypes (53). Thus, we

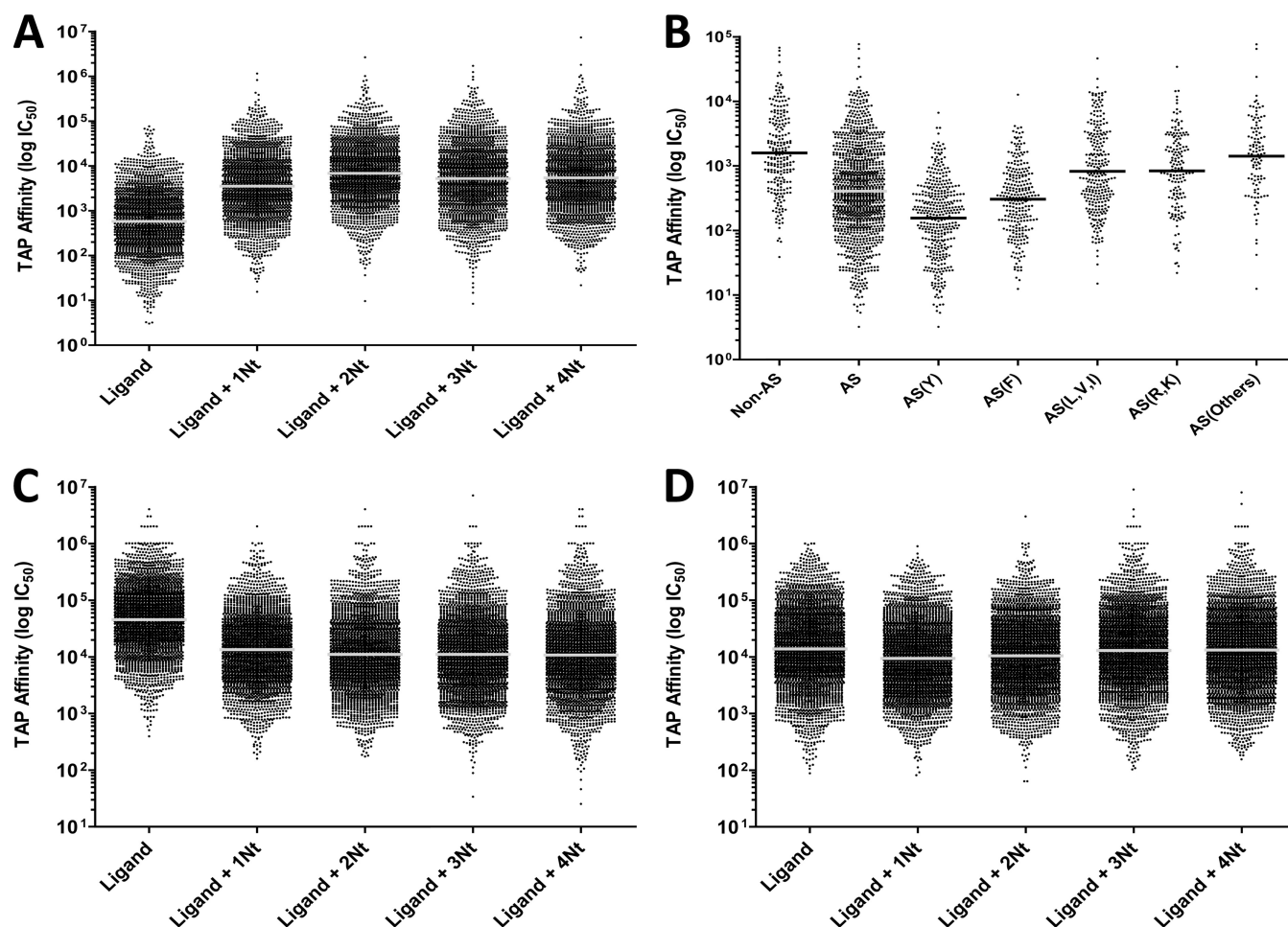


FIG. 6. Predicted affinity of HLA-B27 and other ligands and their precursors for TAP. A, TAP binding affinity of the natural HLA-B27 ligands in supplemental Table S2 and their N-terminally extended precursors (Nt) by 1 to 4 residues. Bars indicate the geometrical mean values of each peptide set. Affinity was calculated as previously described (43). The significance of the differences among peptide sets was assessed by the Mann-Whitney test. Natural ligands were better suited to TAP than any of their precursors ($p < 0.0001$), followed by single-residue extended precursors. B, TAP binding affinity of peptides selectively found among non-AS- or AS-associated subtypes. The latter peptide set was subdivided according to the C-terminal residue as indicated. Peptides from AS-associated subtypes were more adapted to TAP ($p < 0.0001$). Among these, peptides with C-terminal aromatic residues were most suitable ($p < 0.0001$), but peptides with C-terminal aliphatic or basic residues were also better TAP binders than non-AS-associated peptides ($p < 0.0001$). C, TAP binding affinity of HLA-B*07:02 ligands ($n = 3214$) and their precursors. Conventions are as in panel A. D, TAP binding affinity of HLA-A*02:01 ligands ($n = 3502$) and their precursors. Conventions are as in panel A. The data in panels C and D are based on previously published sequence databases (45) and are included here only for comparison. Reported sequences assigned only to HLA-B7 or HLA-A2 were included.

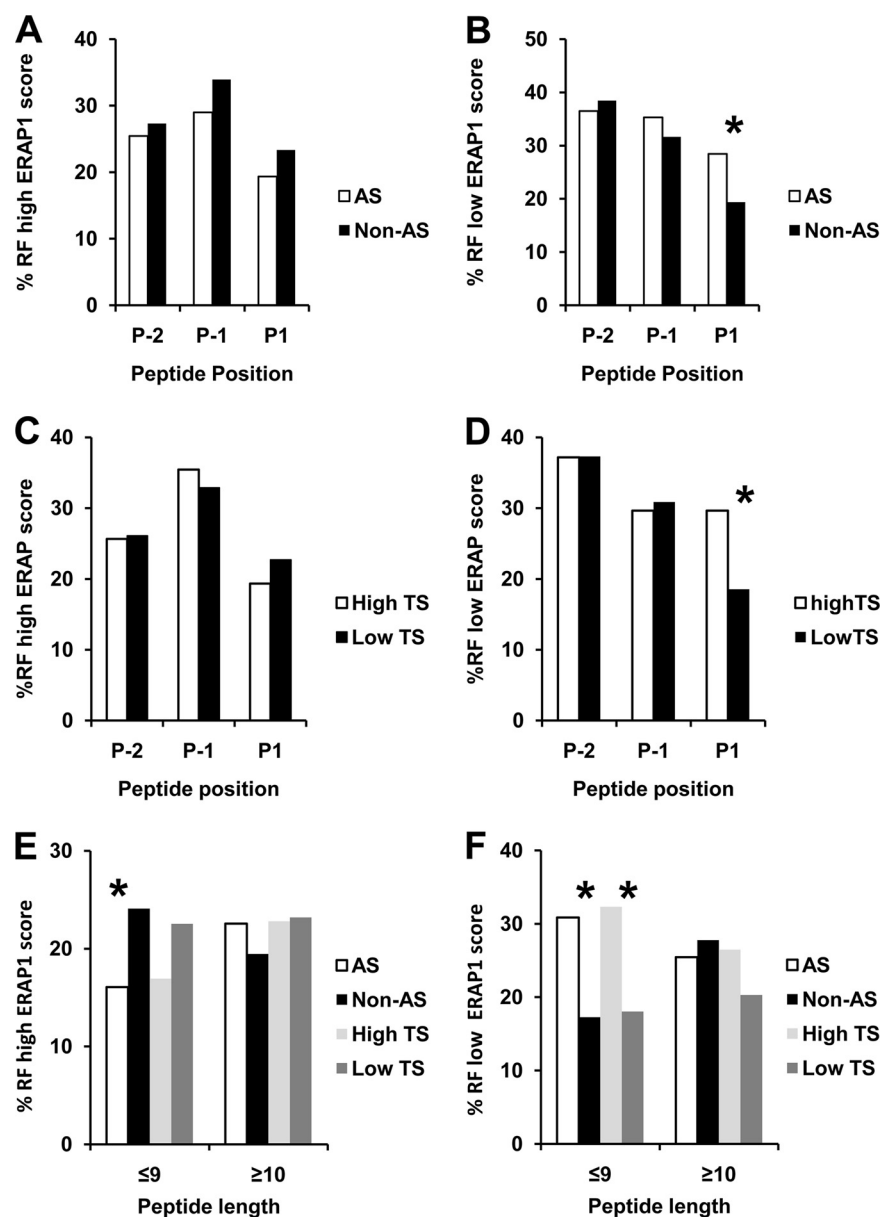
compared the global susceptibility to ERAP1 of the two N-terminal flanking (P-2 and P-1) and the P1 residues of peptide ligands found only among AS-associated subtypes with those found only in non-AS-associated ones. This comparison focused on the frequency of the residues highly susceptible to ERAP1 (score >50 : Y, M, L, A, and C) and those with the lowest susceptibility (score <10 : E, D, V, W, K, R, and P). The joint frequency of susceptible flanking and P1 residues was similar in both peptide subsets (Fig. 7A). However, peptides found only in AS-associated subtypes showed a statistically significant increase of ERAP1-resistant P1 residues relative to non-AS-associated subtypes (Fig. 7B). When the same analysis was carried out for peptides selectively found among either high or low thermostability subtypes a similar tendency

was found (Fig. 7C–7D). These differences were due mainly to the 9-mers and shorter peptides (Fig. 7E–7F).

In addition, there was an increased frequency of resistant versus susceptible P1 residues among peptides selectively found in AS-associated and high thermostability subtypes, (ratio 1.5). The differences were increased (ratio 1.9) when only peptides ≤ 9 -mers were considered. In contrast, peptides from non-AS-associated or low thermostability subtypes, or the corresponding subsets of peptides ≤ 9 -mers, showed no increased frequencies (ratio 0.7–0.8) of resistant versus susceptible P1 residues (Table V).

Taken together, these results reveal a differential influence of ERAP1 on AS-associated/high-thermostability and non-AS-associated/low-thermostability subtypes.

FIG. 7. ERAP1 susceptibility of the flanking and P1 residues of peptides selectively found among HLA-B27 subtypes with differential AS-association or thermostability. A, Joint frequency of highly susceptible residues (score > 50) at the P-2, P-1, and P1 positions in the AS-associated (white bars) and the non-AS-associated subtype ligands (black bars). B, Joint frequency of low susceptibility residues (score < 10) at the P-2, P-1, and P1 positions in the AS-associated (white bars) and the non-AS-associated subtype ligands (black bars). Statistically significant differences ($p = 6.2 \times 10^{-3}$) are indicated (*). (C) Joint frequency of susceptible residues at the P-2, P-1, and P1 positions in the high (white bars) and low thermostability subtype ligands (black bars). D, Joint frequency of low susceptibility residues at the P-2, P-1, and P1 positions in the high (white bars) and low thermostability subtype ligands (black bars). Statistically significant differences are indicated ($p = 1.4 \times 10^{-4}$). E, Joint frequency of susceptible P1 residues of the peptides selectively found among AS-associated (white bars), non-AS-associated (black bars), high thermostability (light gray bars), or low thermostability (dark gray bars) subtypes. Peptides were classified by length in ≤ 9 -mers and ≥ 10 -mers. Statistically significant differences are indicated ($p = 0.016$). F, Joint frequency of low susceptibility P1 residues of the same peptide subsets as in panel E. Statistically significant differences ($p = 3.5 \times 10^{-4}$ and 5.6×10^{-5} , respectively) are indicated.



DISCUSSION

The present study is, by far, the most extensive analysis of HLA-B27 subtype-bound peptidomes reported to date and allowed us, for the first time, to define the relationship between peptide specificity and other pathogenetic features of this molecule. Because subtype-bound peptides were identified with various methods and compiled together in a single database there is a heterogeneity that is likely to affect the completeness of subtype assignments. It is obvious that peptides not found in a given subtype cannot be excluded as ligands of that subtype. A more uniform and sensitive methodology would certainly show higher peptide sharing among subtypes, which should then require a quantitative assessment, because shared peptides can be presented by different subtypes at widely different levels. A quantitative comparison

of subtype-bound peptidomes was not the purpose and is out of the scope of this study.

An important issue is the use of a 5% FDR threshold for peptide assignments. It can be argued that it is a rather relaxed one and may introduce an excessive number of false positives. It has to be noted, however, that in MHC peptidomics MS/MS searches are typically conducted without enzyme restriction. This leads to a huge increase of the search space compared with a standard tryptic workflow. Under these circumstances, the chances of random matching increase and high-scoring decoys lead to a huge loss in sensitivity if peptide matches are filtered at 1% FDR, an issue discussed at length elsewhere (45). Additionally, in this scenario, a FDR of 1% may be reached with a very low number of decoy hits, compromising the accuracy of FDR estimations.

TABLE V

ERAP1 susceptibility of N-terminal residues selectively found among HLA-B27 subtypes with differential AS association or thermostability. The data represent the mean frequency (%) of residues with trimming susceptibility scores <10 (resistant), >50 (susceptible), and the resistant/susceptible ratio among the peptides selectively found among the AS-associated, non-AS-associated, high thermostability, low thermostability subtypes, and the corresponding subsets of peptides ≤9-mers. The ratios reflecting an increased frequency of resistant vs. susceptible residues are highlighted in boldface

	N ^a	Resistant (%)	Susceptible (%)	Ratio
AS association				
AS-associated	1167	28.4	19.4	1.5
≤9-mers	616	30.8	16.1	1.9
Non-AS associated	227	19.4	23.3	0.8
≤9-mers	191	17.3	24.1	0.7
Thermostability				
High	988	29.7	19.3	1.5
≤9-mers	526	32.3	16.9	1.9
Low	313	18.5	22.7	0.8
≤9-mers	244	18.0	22.5	0.8

^a Number of peptides (7- to 15-mers).

For these reasons FDR filters are typically less stringent, and a 5% FDR is currently standard, in MHC peptidomics. We are aware that this threshold is rather lean and will introduce a certain fraction of false positives. Nevertheless, this should be largely compensated by avoiding the great loss of true positives occurring at more stringent FDR. Improvements in the accuracy of MS instruments, and in search and scoring algorithms, should facilitate in a near future a more robust FDR assessment, in line with that of trypsin-based proteomics.

With the above limitations in mind, HLA-B27 subtypes showed three peptide specificity patterns according to their preferences for C-terminal residues: (1) B*27:03 and B*27:05 preferentially bound peptides with basic, aliphatic and aromatic residues, (2) B*27:02 and B*27:04 were restricted to C-terminal aliphatic/aromatic residues with a low allowance for basic ones, and (3) B*27:06, 07, and 09 were restricted to nonpolar aliphatic and F residues, almost excluding Y. Subtypes from groups 1 and 2 showed a preference for large C-terminal residues, whereas those from group 3 preferred smaller ones. The exclusion of polar and large C-terminal residues in B*27:06, 07, and 09 is because of the substitution of D116 in these subtypes for non-acidic and bulkier residues (Y, H) that decrease the polarity and size of the F pocket. The low preference for C-terminal basic residues, but not Y, in B*27:02 and 04 is presumably determined by the change of D77 to small polar residues (N, S) in these subtypes (supplemental Table S1).

The comparable peptide sharing among subtypes binding either very distinct (i.e. B*27:05/07) or similar (i.e. B*27:06/09) C-terminal residues indicates that differences at other peptide positions, which are determined by polymorphism outside the F pocket, introduce significant diversity among subtype-

bound peptidomes. For instance, the increased frequencies of basic P3 and PC-2 residues, and Y3, in B*27:04 and B*27:06, are presumably because of the V152E change in both subtypes and to the H114D change in B*27:06. The high frequency of N-terminal basic residues in B*27:03, which extends previous observations (22, 23), is thought to be a compensatory effect of interactions between basic P1 side chains and E163, for the altered hydrogen bonding of the peptidic N terminus in the A pocket of this subtype (54).

The relationship between the peptidomes and other features of HLA-B27 subtypes revealed the key role of residue 116 in unifying HLA-B27 biology. Among subtypes with identical A and B pockets, absence of D116 correlated strictly with decreased molecular stability, fast folding and almost exclusive binding of peptides with C-terminal nonpolar residues. This is likely the result of an increased hydrophobicity of the F pocket because of abrogation of the acidic charge. The thermodynamic requirements to hide a hydrophobic F pocket from water during the biosynthesis of HLA-B27 would promote fast peptide binding, decrease tapasin dependence, as shown for B*27:06 (55), and lead to fast folding with a sub-optimal peptide repertoire. A similar situation was observed among HLA-B44 subtypes (56).

The correspondence of D116/slow folding/high thermostability with AS is incomplete because B*27:07 lacks D116 and shows fast folding and decreased thermostability (57, 58). Yet, the features of the peptides segregating with AS susceptibility or differential thermostability were very similar. This might be related the fact that the B*27:07 has a comparable peptide sharing with B*27:05 and B*27:06/B*27:09. Like B*27:07, B*14:03, which is associated with AS in African populations (19, 59, 60), has lower thermostability than AS-associated HLA-B27 subtypes and equally low as B*14:02, which is not associated with AS (61). The F pocket of B*14:03 is also hydrophobic and almost exclusively binds peptides with non-polar C-terminal residues (62). Thus, the association of B*27:07 and B*14:03 with AS cannot be explained just as a consequence of their folding and stability features, and its basis remains unknown. This conclusion raises the possibility that, in a more general way, the association of HLA-B27 with AS may be dependent on additional peptide-dependent mechanisms besides those based on folding and stability.

The small number of peptides found in all four AS-associated subtypes and not otherwise might be compatible with a contribution of specific peptides to AS pathogenesis. Yet, the many more peptides found only among AS-associated subtypes, but with diverse distribution patterns, are presumably more relevant because they could globally affect other features of the molecule, such as folding and stability.

It was previously noted that HLA-B27 ligands are particularly well suited to TAP preferences (63). Now we showed that these ligands, irrespective of the subtype(s) in which they were found, are better suited to TAP than their respective N-terminally extended precursors. In addition, peptides with

N-terminal dibasic sequences, which are frequent in HLA-B27, are relatively resistant to cytosolic aminopeptidases (64). Thus, HLA-B27 is particularly well suited to bind peptides directly produced in the cytosol. The observation that HLA-B27 ligands selectively found among AS-associated subtypes were better suited to TAP than those from non-AS-associated ones raises the possibility of a relationship between the direct import of fully processed HLA-B27 ligands from the cytosol and AS association, and is consistent with the reported involvement of the cytosolic puromycin-sensitive aminopeptidase in AS (65). Yet, the significant influence of ERAP1 on the HLA-B27 peptidome (15, 66) implies that many natural ligands are generated and/or destroyed in the ER.

Two features distinguishing the peptides selectively found among AS-associated/high thermostability or non-AS-associated/low thermostability subtypes revealed a differential influence of ERAP1 on both subtype groups: (1) the distinct susceptibility of their P1 residues to ERAP1 trimming, and (2) their distinct length distribution. As discussed above, the fast folding and export rates of non-AS-associated/low-thermostability subtypes presumably result from their fast and sub-optimal loading of peptides in the ER. Thus, these subtypes could load peptides with both short and long half-life, depending on their affinity and abundance. Because the half-life of peptides in the ER is strongly influenced by the susceptibility of their P1 residues to ERAP1, this explains that resistant and susceptible P1 residues are similarly frequent among the peptides selectively found in these subtypes. Moreover, in the absence of tapasin-mediated editing, peptides with optimal length might be preferred, which explains the prevalence of 9-mers in these subtypes.

In contrast, AS-associated/high-thermostability subtypes fold more slowly and bind more optimized peptidomes (57, 58) after tapasin editing (55, 56). Thus, they may preferentially bind peptides with longer half-life in the ER, which are those with P1 residues more resistant to ERAP1, in agreement with our results. Peptide editing would also allow selecting longer peptides if these bind with higher stability than 9-mers, resulting in a more balanced size distribution within the range compatible with MHC-I binding, as also observed here.

The previous observation that ERAP1 knockdown affected the expression levels of AS-associated, but not of non-AS-associated subtypes at the cell surface (53) is most likely explained by the differences in stability and ERAP1 dependence of these subtypes.

Our observations suggest a differential effect of ERAP1 polymorphism on AS- and non-AS-associated subtypes. We previously reported that B*27:04 ligands prevalent in the context of a highly active ERAP1 variant had an increased frequency of ERAP1-resistant P1 residues, relative to the predominant peptides expressed in a less active ERAP1 context (15). This was interpreted as the result of a more extensive destruction of ligands with susceptible P1 residues, relative to those with resistant ones. Thus, the lower discrimination of

non-AS-associated/low-thermostability subtypes for peptides differing in the susceptibility of their P1 residues to ERAP1 suggests that these subtypes may be less influenced by ERAP1 polymorphism than AS-associated/high-thermostability ones. If so, AS-associated ERAP1 alleles might not favor disease development in B*27:06 and B*27:09 positive individuals.

In conclusion, the extensive characterization of HLA-B27 subtype-bound peptidomes reveals the structural basis of their peptide specificity and its close relationship to folding, stability and association with AS. Residue 116 is the key that interconnects all the molecular features of HLA-B27, but other polymorphisms significantly influence the diversity of subtype-bound peptide repertoires. D116 confers an increased polarity to the F pocket and a higher plasticity in its interaction with chemically diverse and bulkier C-terminal peptide residues. These features allow for a proper optimization of the peptidome through tapasin-mediated editing (56), leading to highly stable HLA-B27 molecules and a significant dependence on ERAP1 and its functional polymorphism. Optimization of the peptide cargo is achieved through slow folding, a byproduct of which is increased heavy chain misfolding (67, 68). This behavior would be critical for the disease-predisposing capacity of HLA-B27 and explains that subtypes lacking D116 are biochemically distinct and, except B*27:07, not associated with AS. The pathogenetic relevance of heavy chain homodimers (69), whose expression at the cell surface presumably follows endosomal dissociation of the heterodimer/peptide complexes (4), might seem difficult to reconcile with the lower thermostability of non-AS-associated subtypes, unless one assumes that the longer half-life and/or increased endosomal recycling of high-thermostability subtypes might favor homodimer formation. That B*27:09 shows less surface homodimers than B*27:05 (70) is consistent with this possibility. Thus, the close relationship between peptide specificity, folding, and stability unifies the biology of HLA-B27 and makes the peptidome, its alterations by subtype polymorphism and the differential ERAP1 dependence of subtype-bound peptidomes, critical features of the pathogenetic role of HLA-B27 in AS.

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§ This article contains supplemental Figs. S1 and S2 and Tables S1 to S8.

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